

### **IN THE SPECIFICATION**

Please amend the specification as follows.

Page 5, delete the second paragraph starting at line 5:

~~In a variation of the above-described methods of the invention, the control sample is substituted by a second test sample comprising neutrophils derived from a second individual, the tests samples being taken from individuals at the same time point before, during or after subjection to different regimes which it is desired to compare as stressors, e.g. before, during or after different protocols for medical treatment. In this case, superoxide production above basal for each sample will be corrected with reference to the white cell count or neutrophil count. This gives a measure of coping capacity standardised to allow for differences in the number of white cells in the peripheral blood of the selected individuals and thereby enables quantitative comparison of the stress-inducing effect of the two regimes. Such comparison may be carried out at more than one time point and generally samples from a number of individuals subjected to the two different regimes will be tested. Application of such methodology to compare perceived stress and measured decrease of leucocyte coping capacity associated with elective and acute cardio-pulmonary bypass surgery is detailed in Example 6. Such methodology of the invention has many applications for optimising procedures to minimise psychological stress and provides a highly convenient quantitative method for evaluating proposals for alleviating psychological stress associated with procedures known to be stressful to human, non-human mammals and birds.~~

Page 7, delete the second paragraph starting at line 5:

~~Example 6 describes in more detail the study indicated above comparing perceived stress and leucocyte coping capacity in two groups of patients, elective patients and emergency (acute) patients pre and post cardio-pulmonary bypass surgery. The data for leucocyte coping capacity at 35 minutes post challenge, obtained using whole blood samples and chemiluminescence measurement, is presented in box plots in Figures 6~~

~~and 7 wherein phagocytic capacity per cell per min represents measured relative light units divided by the white cell count and the number of minutes post challenge with adjustment for background activity.~~

Page 7, replace the third paragraph starting at line 17 with the following:

A method of the invention may be carried out on neutrophils obtained from an individual in any manner whereby the neutrophils are in a whole blood sample suitable for chemical-induced superoxide production ~~but will most preferably for convenience be applied to neutrophils in a whole blood sample.~~ For example, in the case of a human, such a sample may be obtained by a simple finger prick. If need be, whole blood samples for use in a method of the invention may be treated with an anticoagulant. ~~While a~~ A whole blood sample may be utilised directly, with or without an anticoagulant, it will be appreciated that a blood fraction comprising neutrophils may alternatively be employed if desired. For example, a sample comprising isolated leucocytes may be employed.

Page 14, delete the second paragraph starting at line 12:

~~As indicated above, where it is desired to compare two regimes as stressors, e.g. two protocols for medical treatment or a known stressful regime plus and minus a proposed means of alleviating stress, then it may be chosen to directly compare coping capacity determined in test samples taken from individuals before, during and after subjection to the two regimes of concern after correction for the number of white cells or neutrophils in each sample.~~

Page 28, delete the second paragraph starting at line 5:

**Example 6 – Evaluation of patients' psychological perceptions and immunological responses pre and post cardio-pulmonary bypass surgery (CPB) in two groups of patients a) those who have waited for elective surgery b) patients who are admitted on an emergency (acute) basis.**

Page 28, delete the third paragraph starting at line 10:

## **Methods**

### **~~(a) Study design~~**

~~Ninety patients who were to undergo CPB were assigned to one of two groups. An 'elective' group n = 64 (51 males, 13 females) who had been on waiting list and an 'acute' group n = 26 (22 males, 4 females). Patients were tested on the evening before surgery and at clinic six or seven weeks after discharge using standard measures to assess psychological well-being. Small peripheral blood samples were taken. Neutrophils' 'Current' activity was determined from whole bloods' capacity to reduce nitro-blue tetrazolium (NBT) (%NBT positive cells), and leukocyte coping capacity, from stimulated whole blood, luminol-dependant chemiluminescence.~~

Page 28, delete the fourth paragraph starting at line 21:

~~Research was approved by the local health authority ethics committee (University Hospitals Coventry and Warwickshire, NHS Trust), the Walsgrave Hospital Research and Development Committee, the School of Science and the Environment ethics committee (Coventry University) and Cardiothoracic consultant Walsgrave hospital, whose patients formed the basis of this research.~~

Page 28, delete the fifth paragraph starting at line 27:

~~The 90 adults in the study underwent cardiopulmonary bypass surgery for either coronary artery bypass grafting (CABG), mitral valve (MV) surgery or aortic valve (AV) surgery and were recruited on the night prior to surgery. The inclusion criteria were:~~

- ~~• Ability to communicate in both spoken and written English language;~~
- ~~• Informed consent;~~
- ~~• The patient was not involved in any other research or experimental procedures.~~

~~The exclusion criteria were:~~

• ~~Previous coronary bypass grafting;~~

• ~~An immune deficiency disorder;~~

~~An inflammatory condition not related to the heart problem.~~

Page 29, delete the second paragraph starting at line 9:

~~The males of the elected group ranged in age from 38 to 80 (mean 63.61, SD 8.75) and the females of the same group ranged in age from 38 to 81 (mean 64.86, SD 13.29). In the acute patient group, the 22 males were aged between 24 and 80 years (mean 64.86, SD 13.29) and the 4 females were aged between 60 and 81 years (mean 71.75, SD 8.85).~~

Page 29, delete the third paragraph starting at line 15:

~~Table 4 below shows a comparison of the patient profiles of the two groups observed. The elective group was reduced to 62 patients post-surgery due to death of one patient and one patient still being in hospital with neurological complications. The acute group was reduced to 20 post-surgery due to the death of 5 patients in hospital and one patient opting out of surgery.~~

Page 30, delete the fourth table starting at line 1:

Table 4: ~~Preoperative comparative patient profile of both groups.~~

	<b>Elective Group</b>		<b>Acute Group</b>	
	<b>Males</b>	<b>Females</b>	<b>Males</b>	<b>Females</b>
Number	<b>51</b>	<b>13</b>	<b>22</b>	<b>4</b>
Age				
Range	<del>38–80</del>	<del>38–81</del>	<del>24–80</del>	<del>60–81</del>
Mean (SD)	<del>63.61 (8.75)</del>	<del>66.62 (10.67)</del>	<del>64.86 (13.29)</del>	<del>71.75 (8.85)</del>
Type of Surgery				
CABG	<del>[[37]]</del>	<del>[[4]]</del>	<del>[[12]]</del>	<del>[[3]]</del>

Valve	[[10]]	[[8]]	[[6]]	[[1]]
CABG & Valve	[[3]]	[[1]]	[[4]]	
CABG, Valve & other	[[1]]			
Angina Status				
Asymptomatic	[[6]]	[[4]]	[[6]]	
NYHA 1	[[7]]	[[2]]		
NYHA 2	[[14]]	[[2]]	[[1]]	
NYHA 3	[[20]]	[[3]]	[[3]]	[[1]]
NYHA 4	[[4]]	[[2]]	[[12]]	[[3]]
Dyspnoea Status				
Asymptomatic	[[7]]		[[1]]	
NYHA 1	[[11]]		[[2]]	
NYHA 2	[[14]]	[[2]]	[[4]]	
NYHA 3	[[11]]	[[4]]	[[4]]	[[2]]
NYHA 4	[[8]]	[[7]]	[[11]]	[[2]]
Coronary Disease				
1 Vessel	[[3]]			
2 Vessel	[[9]]	[[2]]	[[1]]	
3 Vessel	[[30]]	[[3]]	[[15]]	[[2]]
N/A Valve only	[[9]]	[[8]]	[[6]]	[[2]]
ASA Grade				
Healthy	[[3]]			
Mild	[[11]]	[[1]]	[[2]]	
Severe	[[32]]	[[11]]	[[11]]	[[3]]
Severe/Life threatening	[[5]]	[[1]]	[[9]]	[[1]]
Previous Infarct				
None	[[30]]	[[11]]	[[8]]	[[2]]
Single	[[15]]	[[2]]	[[10]]	[[2]]
Multiple	[[6]]		[[4]]	
Post Surgery Status				

Alive	[[49]]	[[13]]	[[18]]	[[2]]
Still hospitalized	[[1]]			
Deceased in Hospital	[[1]]		[[4]]	[[1]]
Opted out/surgery				[[1]]

Page 31, delete the fourth footnote starting at line 1:

Key To Table 4

GABG = Coronary Artery Bypass Grafting

Valve = Mitral or Aortic Valve replacement or repair

The physicians and the surgeons, on the following scale, preoperatively, graded both angina and dyspnoea status:

NYHA 1 = No limitations of normal physical activity

NYHA 2 = Ordinary physical activity causes discomfort / dyspnoea

NYHA 3 = Moderate to great limitations of ordinary physical activity

NYHA 4 = Unable to perform any physical activity without discomfort / dyspnoea

As a gross predictor of overall outcome, post-operatively, an anaesthetist graded each patient on the evening before surgery, using what is termed the ASA grade, as follows:

ASA I = Healthy patient

ASA II = Mild systemic disease — no functional limitation

ASA III = Severe systemic disease — definite functional limitation

ASA IV = Severe systemic disease that is a constant threat to life

Page 31, delete the first paragraph starting at line 18:

***(b) Procedure***

Patients were approached by the researcher upon their arrival at hospital or on the evening prior to surgery (often one and the same time). Briefly, patients were told that

~~the research was to measure their levels of perceived stress and anxiety and the effects this may have on neutrophils before and after surgery. They were additionally told that they would be required to complete a number of simple questionnaires and to provide a small sample of blood from a finger (the equivalent of three drops) on two occasions, the first being during that evening and the second at the post-operative clinic approximately six weeks after surgery. Any questions were answered and an information sheet was given to each patient and any relatives in attendance. The researcher then gave the patients time to think about this, returning later with consent forms and asking again if they were willing to take part, explaining that they could if they wished withdraw at any time. If consent was given, demographic data was recorded and patients were asked to complete the questionnaires. A finger stick blood sample was taken from a cleaned finger using a Soft-Clix™ and sterile lancet and a Gilson pipette was used to collect the blood (3 by 10 µl samples were taken). One sample was placed in 40 µl of luminol in a luminometer test tube. The second sample was mixed with an equal quantity of nitro-blue tetrazolium (NBT) and the third sample was placed in 90 µl of diluting fluid for white cell counting (see sample protocol below). The wound was dressed if required. Any questions were answered and then the patients were left in peace.~~

Page 32, delete the second paragraph starting at line 13:

~~At the post-operative clinic approximately six to seven weeks after surgery, patients were again seen by the researcher and were asked if they wished to continue. If so, the questionnaires were again completed and blood samples taken again.~~

Page 32, delete the third paragraph starting at line 17:

**~~(c) Protocols~~**

~~(i) Measurement of PMA-challenge-induced superoxide production in blood samples 10 µl of whole blood was mixed with 40 µl of luminol diluted to 10<sup>-4</sup>M with phosphate buffered saline (PBS) in a luminometer test tube. The sample was placed in a luminometer and relative light units (RLU) were recorded after a period of 60 seconds.~~

~~This initial test was carried out to determine the background activity of the sample so that when calculating coping capacity this background activity could be deducted. 20 µl of phorbol-12-myristate-13-acetate (PMA) and 20 µl of formyl-Met-Leu-Phe (fMLP) were then added and RLU were recorded at the end of 5 minutes, 20 minutes and 35 minutes after stimulation. Coping capacity was determined by dividing the RLU by the white cell count and the number of minutes of the test and adjusting for background activity and presented as phagocytic capacity per cell per min.~~

Page 33, delete the first paragraph starting at line 2:

~~(ii) Nitro-Blue-Tetrazolium Test (NBT)~~

~~This test used to determine the 'current' oxidative state of the neutrophils within the sample, as determined by the oxidative capacity of the neutrophils to reduce nitro-blue tetrazolium and form a formazan pigment.~~

Page 33, delete the second paragraph starting at line 7:

~~10 µl of blood was mixed with 10 µl of NBT, diluted at 1mg per ml, and incubated at 37°C for ten minutes sample was then smeared onto two clean microscope slides and air dried. Samples were then flooded with Accustain Wright stain, rinsed with distilled water and dried. Percentage of 'activated' neutrophils was determined by careful counting under the light microscope.~~

Page 33, delete the third paragraph starting at line 13:

~~(iii) Whole-White-Cell Count (WCC)~~

~~A manual WCC count was undertaken for each patient. 10 µl of blood was mixed with 90 µl of 2% acetic acid coloured pale purple with crystal violet and gently mixed to lyse the red blood cells. A sample was then placed on a haemocytometer and a careful count was made. A mean WCC per ml was calculated from at least five fillings of the haemocytometer.~~

Page 33, delete the fourth paragraph starting at line 20:



~~(iv) Measures~~

~~Each patient completed the ten-item Perceived Stress Scale (Cohen, Kamarek & Mermelstein, 1983), the 14-item Hospital Anxiety Depression Scale (Zigmond & Snaith) and the 20-item Positive and Negative Effect Scale (PANS) (Watson, Clark & Tellegen 1988). In addition, a six-item visual analogue scale was constructed to investigate the perception of stress specifically about the illness, the self-efficacy of the patient and their locus of control. The items from this scale are listed below:~~

- ~~1. How 'stressed' do you feel about your health **Right Now?**~~
- ~~2. How much control do you feel you have over your health in general?~~
- ~~3. How much control do you feel you have over your heart condition **Right Now?**~~
- ~~4. Overall how confident are you that your heart condition will have improved in 12 months time?~~
- ~~5. How much control do you feel health professionals have over your heart condition **Right Now?**~~
- ~~6. How confident are you that you can do all the things necessary to manage your condition and/or its treatments on a regular basis?~~

~~Each item could be scored on a scale from 0 (no stress, no control, not confident) to 10 (stress as bad as it can be, complete control, completely confident).~~

Page 34, delete the second paragraph starting at line 12:

~~Clinical pre-operative data was also recorded for all patients including previous infarct; angina status, lung function and grading by the anaesthetist (ASA grade) (data obtained from hospital record database).~~

Page 34, delete the third paragraph starting at line 16:

Results

**(i) Summary**

~~Results revealed a consistent, statistically significant association between stress and %NBT cells pre and post surgery ( $p < 0.001$ ) in both groups. There was a significant difference in perceived stress ( $p = 0.009$ ) between the groups, pre surgery, both~~

~~significantly reduced post-surgery. No significant differences were found between or within the groups for anxiety, depression, and positive and negative effect. Interestingly, the 'acute' group had the highest perceived psychological stress as determined by self-administered questionnaires. However, coping capacity as determined from blood samples was significantly greater in the 'acute' group both before and after surgery ( $p < 0.02$ ).~~

Page 35, delete the first paragraph starting at line 1:

~~(iii) Whole White Cell Count (WCC)~~

~~A manual WCC count was undertaken for each patient. 10  $\mu$ l of blood was mixed with 90  $\mu$ l of 2% acetic acid coloured pale purple with crystal violet and gently mixed to lyse the red blood cells. A sample was then placed on a haemocytometer and a careful count was made. A mean WCC per ml was calculated from at least five fillings of the haemocytometer.~~

Page 35, delete the first paragraph starting at line 1:

~~(ii) Perceived stress, anxiety and the current activity (NBT) of neutrophils.~~

~~(a) Pre-Surgery~~

~~A consistent and significant relationship between PSS and percentage of NBT positive neutrophils was found in both groups ( $r = 0.912$ ,  $p = 0.0001$  in the elective group and  $r = 0.878$ ,  $p = 0.0001$  in the acute group).~~

Page 35, delete the second paragraph starting at line 7:

~~There were also found consistent and significant relationships in both groups between the percentages of NBT positive neutrophils and anxiety ( $r = 0.670$ , &  $r = 0.616$ ,  $p = 0.0001$ ), depression ( $r = 0.632$ , &  $r = 0.664$ ,  $p = 0.0001$ ), negative effect ( $r = 0.601$ ,  $p = 0.0001$  &  $r = 0.581$ ,  $p = 0.001$ ) and dyspnoea status ( $r = 0.275$ ,  $p = 0.028$  &  $r = 0.399$ ,  $p = 0.044$ ). There was also found a significant relationship between NBT and health related stress within the elective group ( $r = 0.499$ ,  $p = 0.0001$ ) but not in the acute group.~~

Page 35, delete the third paragraph starting at line 15:

~~(b) Post Surgery~~

~~A consistent and significant relationship between PSS and percentage of NBT positive neutrophils was found in both groups ( $r = 0.851$ ,  $p = 0.0001$  in the elective group and  $r = 0.944$ ,  $p = 0.0001$  in the acute group.~~

Page 35, delete the fourth paragraph starting at line 20:

~~Consistent and significant relationships were found between the percentage of NBT positive neutrophils and PSS ( $r = 0.851$  &  $r = 0.944$ ,  $p = 0.0001$ ), anxiety ( $r = 0.488$ , &  $0.864$ ,  $p = 0.0001$ ), depression ( $r = 0.475$ ,  $p = 0.0001$  &  $r = 0.688$ ,  $p = 0.001$ ), positive effect ( $r = -0.329$ ,  $p = 0.09$  &  $r = -0.593$ ,  $p = 0.006$ ), negative effect ( $r = 0.494$ , &  $r = 0.831$ ,  $p = 0.0001$ ) and health related stress ( $r = 0.456$ , &  $r = 0.707$ ,  $p = 0.0001$ ).~~

Page 35, delete the fifth paragraph starting at line 27:

~~The above results, from both the pre and post surgery time points, suggest that the percentage of NBT positive neutrophils show considerable associations with the psychological state of the patients studied with the negative states (e.g. anxiety, depression, PSS, negative effect and health related stress) increasing as the percentage of NBT neutrophils increases. Positive effect shows a negative association suggesting that as positive effect increases the percentage of NBT positive neutrophils decreases. However few or no associations are seen between NBT and disease indicators.~~

Page 36, delete the second paragraph starting at line 7:

~~(iii) Coping capacity of neutrophils~~

~~Analysis of the chemiluminescence data (Mann-Whitney U test) comparing the groups at each time-point revealed no significant differences between the groups at 5 minutes or 20 minutes for pre or post surgery, samples (see Tables 5 and 6 below). However, a~~

~~consistent and significant difference in coping capacity was found at 35 minutes for both pre ( $p = 0.018$ ) and post surgery ( $p = 0.019$ ) (see Figures 6 and 7).~~

Page 36, delete the fifth table starting at line 14:

~~Table 5: Comparison of the phagocytic capacity at the three time points (pre-surgery)~~

	Elective Group			Acute Group			
	n = 64			n = 26			
Time	median	max	min	median	max	min	p
5 mins.	0.00743	0.2112	-0.0011	0.00775	0.0194	-0.0027	ns
20 mins.	0.01547	0.0967	0.0017	0.17423	0.0684	0.0026	ns
35 mins.	0.35613	0.5069	0.0065	0.05610	0.1679	0.0153	0.018

Page 36, delete the sixth table starting at line 16:

~~Table 6: Comparison of the phagocytic capacity at the three time points (post-surgery)~~

	Elective Group			Acute Group			
	n = 62			n = 20			
Time	median	max	min	median	max	min	p
5 mins.	0.00191	0.0226	-0.0042	0.00599	0.0123	-0.0011	ns
20 mins.	0.01701	0.0883	-0.0123	0.01954	0.1086	0.0028	ns
35 mins.	0.03299	0.1677	-0.0163	0.04368	0.1137	0.0224	0.019

Page 37, delete the first paragraph starting at line 2:

~~Bivariate correlations of the data show no significant associations between coping capacity and either the psychological variables or the physiological indicators.~~

Page 37, delete the second paragraph starting at line 5:

~~(iv) **Perceived stress and health-related stress.**~~

~~The level of PSS reported in both groups prior to surgery was significantly higher than post surgery ( $t = 2.72, p = 0.008$  &  $t = 3.44, p = 0.001$ ). It is also clear from the mean scores that the acute group were reporting significantly higher levels of PSS than the elective group ( $t = 2.674, p = 0.009$ ). This is also true of health related stress which was significantly higher pre-surgery than post-surgery in both groups ( $t = 5.19, p = 0.000$ , &  $t = 3.76, p = 0.001$ ). Again, the highest mean score is in the acute group pre surgery. There were no significant differences between the health related stress scores between the groups pre-surgery. The 'norm' PSS for this population was  $12 \pm 6.3$  (SD) compared to slightly higher in the elective group ( $14.72, \pm 7.21$ ), but much higher in the acute group ( $19.12, \pm 6.71$ ), suggesting that the acute group was the more 'stressed'. Post surgery scores were near to population 'norms'.~~

Page 37, delete the third paragraph starting at line 19:

~~**(v) Conclusion**~~

~~Whilst patients from the acute group perceived themselves to be more stressed than patients from the elective group, their leucocytes had a greater coping capacity than those from the elective group. In physiological terms they would be less susceptible to opportunistic infections and stress-related illness.~~